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FORM PTO-1390 (REV. 9-2001) U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE

TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371

ATTORNEY 'S DOCKET NUMBER: AM100039

U.S. APPLICATION NO. (If known, see 37 CFR 1.5: 10/019163

INTERNATIONAL APPLICATION NO.	INTERNATIONAL FILING DATE	PRIORITY DATE CLAIMED
PCT/US00/17019	June 20, 2000	June 25, 1999

TITLE OF INVENTION
Extraction of Integral Membrane Proteins

APPLICANT(S) FOR DO/EO/US
Sanjay Lakhotia & Michael R. Biehl

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. [X] This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. [ ] This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. [ ] This is an express request to begin national examination procedures (35 U.S.C. 371(f)). The submission must include items (5), (6), (9) and (21) indicated below.
4. [ ] The US has been elected by the expiration of 19 months from the priority date (Article 31).
5. [X] A copy of the International Application as filed (35 U.S.C. 371(c)(2))
   a. [ ] is attached hereto (required only if not communicated by the International Bureau).
   b. [ ] has been communicated by the International Bureau.
   c. [X] is not required, as the application was filed in the United States Receiving Office (RO/US). (copy of Abstract page 41 is enclosed)
6. [ ] An English language translation of the International Application as filed (35 U.S.C. 371(c)(2)).
   a. [ ] is attached hereto.
   b. [ ] has been previously submitted under 35 U.S.C. 154(d)(4).
7. [ ] Amendments to the claims of the International Aplication under PCT Article 19 (35 U.S.C. 371(c)(3))
   a. [ ] are attached hereto (required only if not communicated by the International Bureau).
   b. [ ] have been communicated by the International Bureau.
   c. [ ] have not been made; however, the time limit for making such amendments has NOT expired.
   d. [ ] have not been made and will not be made.
8. [ ] An English language translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371 (c)(3)).
9. [X] An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)).-Two
10. [ ] An English lanugage translation of the annexes of the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).

Items 11 to 20 below concern document(s) or information included:

11. [ ] An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
12. [ ] An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
13. [ ] A FIRST preliminary amendment.
14. [ ] A SECOND or SUBSEQUENT preliminary amendment.
15. [ ] A substitute specification.
16. [ ] A change of power of attorney and/or address letter.
17. [ ] A computer-readable form of the sequence listing in accordance with PCT Rule 13ter.2 and 35 U.S.C. 1.821 - 1.825.
18. [ ] A second copy of the published international application under 35 U.S.C. 154(d)(4).
19. [ ] A second copy of the English language translation of the international application under 35 U.S.C. 154(d)(4).
20. [X] Other items or information:
    a)International Search Report
    b)International Preliminary Examination Report

CERTIFICATE OF MAILING 37 CFR §1.10

I hereby certify that this paper and the documents referred to as enclosed therein are being deposited with the United States Postal Service on the date written below in an envelope as "Express Mail Post Office to Addressee" Mailing Label Number ET937267973US addressed to the Commissioner for Patents, Washington, DC 20231

12/20/01
Date

Alan M. Gordon
Alan M. Gordon

531 Rec'd PCT/PTO 20 DEC 2001

U.S. APPLICATION NO. (if known, see 37 CFR 1.5) 10/019163	INTERNATIONAL APPLICATION NO. PCT/US00/17019	ATTORNEY'S DOCKET NUMBER AM100039

	CALCULATIONS	PTO USE ONLY
21. [x] The following fees are submitted: **BASIC NATIONAL FEE (37 CFR 1.492 (a) (1) - (5)):** Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO .... **$1040.00** International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO .... **$890.00** International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search fee (37 CFR 1.445(a)(2)) paid to USPTO .... **$740.00** International preliminary examination fee (37 CFR 1.482) paid to USPTO but all claims did not satisfy provisions of PCT Article 33(1)-(4) .... **$710.00** International preliminary examination fee (37 CFR 1.482) paid to USPTO and all claims satisfied provisions of PCT Article 33(1)-(4) **$100.00**		
ENTER APPROPRIATE BASIC FEE AMOUNT =	$ 890.00	
Surcharge of **$130.00** for furnishing the oath or declaration later than [ ] 20 [ ] 30 months from the earliest claimed priority date (37 CFR 1.492(e)).	$	

CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE	$	
Total claims	16 - 20 =	0	x **$18.00**	$ 00.00	
Independent claims	4 - 3 =	1	x **$84.00**	$ 84.00	
MULTIPLE DEPENDENT CLAIM(S) (if applicable)			+ **$280.00**	$	

	CALCULATIONS	PTO USE ONLY
TOTAL OF ABOVE CALCULATIONS =	$ 974.00	
[ ] Applicant claims small entity status. See 37 CFR 1.27. The fees indicated above are reduced by 1/2. +	$	
SUBTOTAL =	$ 974.00	
Processing fee of **$130.00** for furnishing the English translation later than [ ] 20 [ ] 30 months from the earliest claimed priority date (37 CFR 1.492(f)).	$	
TOTAL NATIONAL FEE =	$ 974.00	
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). **$40.00** per property +	$	
TOTAL FEES ENCLOSED =	$ 974.00	
	Amount to be refunded:	$
	charged:	$

a. [ ] A check in the amount of $ ________ to cover the above fees is enclosed.

b. [x] Please charge my Deposit Account No. 01-1300 in the amount of $974.00 to cover the above fees. A duplicate copy of this sheet is enclosed.

c. [x] The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 01-1300. A duplicate copy of this sheet is enclosed.

d. [ ] Fees are to be charged to a credit card. **WARNING:** Information on this form may become public. **Credit card information should not be included on this form.** Provide credit card information and authorization on PTO-2038.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137 (a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO:

Alan M. Gordon
American Home Products Corp.
Patent Law Department
Five Giralda Farms
Madison, NJ 07940
(845) 602-4636

Alan M. Gordon
SIGNATURE

Alan M. Gordon
NAME

30,637
REGISTRATION NUMBER

10/019163

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EXTRACTION OF INTEGRAL MEMBRANE PROTEINS

Field of the Invention

This invention is directed to a process for extracting gram-negative integral membrane proteins from bacteria or bacterial host cells containing a recombinant vector by differential detergent tangential flow diafiltration.

Background of the Invention

Gram-negative bacteria possess both an inner membrane and an outer membrane. Collectively, the proteins contained in these membranes are referred to as integral membrane proteins. Native integral membrane proteins can be extracted from gram-negative bacteria in relatively small quantities. Recombinant expression techniques permit these proteins to be expressed from bacteria in increased quantities.

Small scale batch purification of such native or recombinant integral membrane proteins has involved an extraction step utilizing centrifugation to extract protein from bacterial cell lysate, followed by downstream purification using conventional techniques.

However, centrifugation is not preferred for extraction of such proteins on a larger scale, because it is a cumbersome process. A larger scale extraction process is desirable in order to obtain quantities of proteins sufficient for economical manufacturing.

Thus, there is a need for a process for extracting native or recombinant gram-negative integral membrane proteins which avoids the use of centrifugation and is therefore more amenable to scale-up. Two such proteins are the lipidated outer membrane proteins P4 and P6 of *Haemophilus influenzae*.

Summary of the Invention

Thus, it is an object of this invention to develop a process for extracting native or recombinant gram-negative integral membrane proteins which avoids the use of centrifugation and is therefore more amenable to scale-up.

It is another object of this invention to develop a process for selectively solubilizing inner and outer membrane proteins of gram-negative bacteria.

It is a further object of this invention to develop processes for extracting the lipidated forms of the recombinant *H. influenzae* outer membrane proteins P4 and P6 from *E. coli*.

These and other objects of the invention as discussed below are achieved by processes which utilize differential detergent tangential flow diafiltration and avoid the use of centrifugation. These processes also provide for continuous extraction of desired proteins.

For the extraction of native or recombinantly-expressed, gram-negative inner membrane proteins from bacteria or bacterial host cells containing a recombinant vector, respectively, by differential detergent tangential flow diafiltration, a process is used which comprises:

(a) lysing bacteria or bacterial host cells containing a recombinant vector in a fermentation broth;

(b) diafiltering the lysed fermentation broth from (a) with a buffer which is not retained by the diafiltration membrane, wherein said buffer removes intracellular and extracellular contaminants through the permeate, and

using a chelating agent to prevent proteolysis;

(c) diafiltering the lysate from (b) with a detergent and a buffer which is not retained by the diafiltration membrane, wherein said detergent solubilizes and removes inner membrane proteins, and using a divalent cation to stabilize the outer membrane proteins, thereby preventing their solubilization; and

(d) collecting the inner membrane proteins removed in (c).

For the extraction of native or recombinantly-expressed, gram-negative outer membrane proteins from bacteria or bacterial host cells containing a recombinant vector, respectively, by differential detergent tangential flow diafiltration, a process is used which comprises:

(a) lysing bacteria or bacterial host cells containing a recombinant vector in a fermentation broth;

(b) diafiltering the lysed fermentation broth from (a) with a buffer which is not retained by the diafiltration membrane, wherein said buffer removes intracellular and extracellular contaminants through the permeate, and using a chelating agent to prevent proteolysis;

(c) diafiltering the lysate from (b) with a detegent and a buffer which is not retained by the diafiltration membrane, wherein said detergent solubilizes and removes inner membrane proteins, and using a divalent cation to stabilize the outer membrane proteins, thereby preventing their solubilization;

(d) diafiltering the lysate from (c) with the buffer from (c), and using a divalent cation from (c) in the absence of detergent, in order to reduce the concentration of the detergent from (c);

(e) diafiltering the lysate from (d) with a buffer which is not retained by the diafiltration membrane, a chelating agent and a detergent to solubilize and remove the outer membrane proteins; and

(f) collecting the outer membrane proteins removed in (e).

If desired, further extraction can be performed by adding the following steps to the foregoing process:

(g) diafiltering the lysate from (e) with reagents of (e), with the exception of the detergent, in order to reduce the concentration of the detergent;

(h) diafiltering the lysate from (g) with reagents of (e); and

(i) collecting the outer membrane proteins removed in (h).

For the extraction of lipidated recombinant, outer membrane protein P4 (lipidated rP4) of *H. influenzae* from bacterial host cells by differential detergent tangential flow diafiltration, a process is used which comprises:

(a) lysing bacterial host cells in a fermentation broth;

(b) diafiltering the lysed fermentation broth from (a) with a buffer which is not retained by the diafiltration membrane, wherein said buffer removes intracellular and extracellular contaminants through the permeate, and

using a chelating agent to prevent proteolysis;

(c) diafiltering the lysate from (b) with a detergent and a buffer which is not retained by the diafiltration membrane, wherein said detegent solubilizes and removes inner membrane proteins, and using a divalent cation to stabilize the outer membrane proteins, thereby preventing their solubilization;

(d) diafiltering the lysate from (c) with the buffer from (c), and using a divalent cation from (c) in the absence of detergent, in order to reduce the concentration of the detergent from (c);

(e) diafiltering the lysate from (d) with a buffer which is not retained by the diafiltration membrane, a chelating agent and a detergent to solubilize the outer membrane proteins;

(f) diafiltering the lysate from (e) with a buffer which is not retained by the diafiltration membrane, a chelating agent and a detergent to extract and remove the lipidated rP4; and

(g) collecting the lipidated rP4 removed in (f).

If desired, further extraction of lipidated rP4 can be performed by adding the following steps to the foregoing process:

(h) diafiltering the lysate from (f) with reagents of (f), with the exception of the detergent, in order to reduce the concentration of the detergent;

(i) diafiltering the lysate from (h) with reagents of (f) to extract and remove the lipidated rP4; and

(j) collecting the lipidated rP4 removed in (i).

If also desired, still further extraction of lipidated rP4 can be performed by adding the following steps to the foregoing process:

(k) diafiltering the lysate from (j) with reagents of (f), with the exception of the detergent, in order to reduce the concentration of the detergent;

(l) diafiltering the lysate from (k) with reagents of (f) to extract and remove the lipidated rP4; and

(m) collecting the lipidated rP4 removed in (l).

Still further cycles of lipidated rP4 extraction may be utilized, if desired.

For the extraction of lipidated recombinant, outer membrane protein P6 (lipidated rP6) of *H. influenzae* from bacterial host cells by differential detergent tangential flow diafiltration, a process is used which comprises:

(a) lysing bacterial host cells in a fermentation broth;

(b) diafiltering the lysed fermentation broth from (a) with a buffer which is not retained by the diafiltration membrane, wherein said buffer removes intracellular and extracellular contaminants through the permeate, and using a chelating agent to prevent proteolysis;

(c) diafiltering the lysate from (b) with a detergent and a buffer which is not retained by the diafiltration membrane,

wherein said detergent solubilizes and removes inner membrane proteins, and using a divalent cation to stabilize the outer membrane proteins, thereby preventing their solubilization;

(d) diafiltering the lysate from (c) with a buffer which is not retained by the diafiltration membrane, a chelating agent to sequester divalent cation and to prevent proteolysis, and a detergent to solubilize and remove the outer membrane proteins other than lipidated rP6;

(e) diafiltering the lysate from (d) with a buffer which is not retained by the diafiltration membrane, a chelating agent to prevent proteolysis, a detergent to remove additional outer membrane proteins, and a salt to disrupt the membrane/outer membrane protein complex;

(f) diafiltering the lysate from (e) with reagents of (e), with the exception of the detergent and the salt, in order to reduce the concentration of the detergent;

(g) diafiltering the lysate from (f) with a detergent and a buffer which is not retained by the diafiltration membrane, wherein said detergent solubilizes and removes additional proteins bound to the cellular outer membrane, and using a chelating agent to prevent proteolysis;

(h) diafiltering the lysate from (g) with the buffer from (g) and the chelating agent of (g) to reduce the

concentration of the detergent from (g);

(i) diafiltering the lysate from (h) with a phosphate compound and a detergent to solubilize and remove additional proteins bound to the cellular outer membrane;

(j) diafiltering the lysate from (i) with a phosphate compound to reduce the concentration of the detergent from (i);

(k) heating the lysate from (j) to remove lipidated rP6 from the membrane while diafiltering that lysate with a phosphate compound and a detergent to solubilize, extract and remove the lipidated rP6; and

(l) collecting the lipidated rP6 removed in (k).

If desired, the process for extracting lipidated rP6 may be modified by concentrating the lysate from (j) before proceeding to (k).

Brief Description of the Figures

Figure 1 depicts an SDS-PAGE analysis of samples taken from the permeate streams during the extraction process for lipidated rP4, as described in Example 1 below. Lanes: 1 - Pharmacia low molecular weight markers; 2 - 0.1 μg lipidated rP4 standard; 3 - 0.3 μg lipidated rP4 standard; 4 - 1 μg lipidated rP4 standard; 5 - Permeate from diafiltration with lysis buffer (10 mM Hepes, 1 mM EDTA); 6 - Permeate from diafiltration with Triton™ X-100; 7 - Permeate from diafiltration with Tris™ buffer; 8 - Permeate from 1x diafiltration with Zwittergent™ 3-12 buffer; 9 -

Permeate from 10x diafiltration with Zwittergent™ 3-12.

Figure 2 depicts the permeate flux from four runs over the time course of the extraction process for lipidated rP4, as described in Example 2 below. The flux is measured in liters/meters^2 membrane area/hour (lmh).

Figure 3 depicts an SDS-PAGE analysis of samples taken from the permeate streams during the first part of the extraction process for lipidated rP6, as described in Example 3 below. Lanes: 1 - Mark 12 standard; 2 - permeate from diafiltration with lysis buffer; 3 - Permeate from diafiltration with Triton™ X-100; 4 - Permeate from diafiltration with Tris™ buffer; 5 - Permeate from diafiltration with Zwittergent™ 3-14; 6 - Permeate from diafiltration with Zwittergent™ 3-14/0.5 M NaCl; 7 - Permeate from diafiltration with Tris™ buffer; 8 - Permeate from diafiltration with Sarcosyl.

Figure 4 depicts an SDS-PAGE analysis of samples taken from the permeate streams during the second part of the extraction process for lipidated rP6, as described in Example 3 below. Lanes: 1 - Mark 12 Standard; 2 - Permeate from diafiltration with Tris™ buffer; 3 -Permeate from diafiltration with Zwittergent™ 3-12 at room temperature; 4 - Permeate from diafiltration with Tris™ buffer; 5 - Permeate from concentration step; 6 - Permeate from diafiltration with Zwittergent™ 3-12 at 55°C; 7 - Permeate from diafiltration with Tris™ buffer at 55°C; 8 - Permeate from diafiltration with Zwittergent™ 3-12 at 55 °C.

Detailed Description of the Invention

This process of this invention for extracting integral membrane proteins has several

advantages over alternate processes. First, this process combines the clarification and extraction processes into one unit operation. The product is extracted from the cells and it is separated from cell debris with only one continuous diafiltration process. Alternative approaches typically require one unit operation for extraction and a second unit operation for clarification. The second advantage is that the membrane proteins are extracted in a semi-purified state, which simplifies the downstream processing steps. Finally, this process is very scalable because the only requirement is that the surface area of the membranes be increased proportionally with the amount of cells.

Prior to the commencement of the extraction process of this invention, an integral membrane protein from a gram-negative bacterium is expressed in a homologous or heterologous bacterial host cell by conventional methods, or the native bacterium is isolated. The fermentation broth is then lysed by passing through a homogenizer to commence the extraction process. In a preferred embodiment of this invention, the homogenizer is a microfluidizer.

The lysed fermentation broth is then subjected to a differential detergent extraction process utilizing tangential flow filtration technology. In this process the lysed cells are diafiltered with a specific sequence of buffer solutions using a tangential flow system that includes a permeable membrane with a defined size cut-off or opening. The sequence of buffer solutions is chosen to solubilize inner membrane proteins first and then to solubilize the outer membrane proteins. During diafiltration, the solubilized proteins of approximate size less than the molecular weight cut-off of the membrane pass through with the permeate, while larger molecules and unsolubilized proteins are retained.

The buffer solutions are then changed and a detergent is introduced to solubilize and extract outer membrane proteins. The sequence of buffer and detergent steps is controlled to extract the desired outer membrane protein in a selective manner. The extracted protein is then purified by conventional means such as ion exchange chromatography.

Thus, the extraction processes of this invention allow selective solubilization of inner and outer membrane proteins of gram-negative bacteria. Solubilized proteins pass through the ultrafiltration membrane with the permeate, while unsolubilized proteins are retained by the membrane.

The native integral membrane proteins extracted using this inventive process are extracted from any suitable gram-negative bacterium, including, but not limited to, *Haemophilus influenzae* (for example, the P4 and P6 proteins), *Moraxella catarrhalis* (for example, the UspA1 and UspA2 proteins), and *Neisseria meningitidis* Group B.

The recombinant integral membrane proteins extracted using this inventive process are expressed in any suitable bacterial host cell containing a recombinant vector, which in turn contains a nucleotide sequence encoding the desired recombinant integral membrane protein. Examples of such bacterial host cells include, but are not limited to, *E. coli*, *Salmonella*, *Shigella* and *B. subtilis*.

Native or recombinant proteins which have a large monomeric, multimeric or aggregate size approaching that of the membrane cut-off, should not be extracted with that membrane. However, gram-negative proteins which are expressed as inclusion bodies in *E. coli*, such as gonococcal or meningococcal proteins, may also be extracted by this process. The inclusion bodies are larger than the membrane cut-off size and are thus retained by the membrane, while

other proteins are extracted. Urea or a similar denaturing agent is then added to solubilize the inclusion bodies. The desired proteins are then extracted and renatured and purified by conventional means.

The extraction process of this invention is exemplified with the recombinant forms of the P4 and P6 proteins of *Haemophilus influenzae*, as expressed in an *E. coli* host cell.

The P4 protein (also known as protein "e") of *Haemophilus influenzae* has a molecular weight of approximately 30 kD and is described in United States Patent 5,601,831, which is hereby incorporated by reference. In its native form, the P4 protein is lipidated. In order to recombinantly express the lipidated P4 protein, the P4 gene is obtained from the bacterium and inserted into an appropriate expression vector. In examples 1 and 2 below, the expression vector pBAD18-Cm (Guzman, L.-M., et al., J. Bacteriol., 177, 4121-4130 (1995)) was used. This vector contains an arabinose inducible promoter and other appropriate control elements. The expression vector is then inserted into a suitable bacterial host cell. In examples 1 and 2 below, the host cell was the *E. coli* BLR strain (Novagen, Madison, WI). If an inducible promoter is used, an inducer is added to cause the host cell to express the desired protein. In examples 1 and 2 below, the inducer was L-arabinose.

The P6 protein (also known as PBOMP-1 and PAL) of *Haemophilus influenzae* has a molecular weight of approximately 15 kD and is described in United States Patent 5,110,908, which is hereby incorporated by reference. In its native form, the P4 protein is lipidated. However, previous attempts to recombinantly express lipidated rP4 resulted in low levels of expression. Copending, commonly-assigned

United States Provisional Patent Application Number 60/141,067 describes an expression system which produces lipidated rP6. In order to recombinantly express the lipidated rP6 protein, the P6 gene is obtained from the bacterium and inserted into an appropriate expression vector. In examples 3 and 4 below, the expression vector pBAD18-CM was again used. The expression vector is then inserted into a suitable bacterial host cell. In examples 3 and 4 below, the host cell was again the *E. coli* BLR strain. If an inducible promoter is used, an inducer is added to cause the host cell to express the desired protein. In examples 3 and 4 below, the inducer was L-arabinose.

In a preferred embodiment of this invention, the diafiltration membrane is from Millipore (Bedford, MA). This membrane is made from regenerated cellulose, has a 1000 kD size cut-off and has a surface area of 0.002 m^2/g wet weight cells.

Any protein-solubilizing detergent may be used in the extraction process including, without limitation, a Zwittergent™ compound such as Zwittergent™ 3-12 or Zwittergent™ 3-14, a non-ionic Triton™ compound such as Triton™ X-100, sarcosyl, a glucoside such as octyl-glucoside, nonyl-glucoside or decyl-glucoside, cholate or deoxycholate, or dodecyl-maltoside. In preferred embodiments, the detergents are Zwittergent™ 3-12, Triton™ X-100 and sarcosyl for the specific steps described herein.

A wide variety of compounds may be used as buffers in the extraction process, as long as the compound is not retained by the diafiltration membrane. Such buffers include, but are not limited to, Hepes, 3-(N-morpholino)propane sulfonic acid (MOPS), Tris™, sodium phosphate and sodium borate. In preferred embodiments, the buffers are Hepes, Tris™

and sodium phosphate for the specific steps desribed herein.

Chelating agents are used at various steps of the extraction process to prevent proteolysis and/or to sequester divalent cations. The preferred chelating agent is EDTA. Divalent cations are used at various steps of the extraction process to stabilize or to solubilize the outer membrane proteins. Divalent cations include metal ions such as Mg^{+2} and Ca^{+2}, with Mg^{+2} being preferred. Sodium chloride is the preferred salt in the salt disruption step in the process for extracting lipidated rP6.

The extraction may be modified by including at least one unit operation with a different diafiltration membrane having a different molecular weight cut-off, such that the lysate passes first through a larger size membrane, and then through least one smaller size membrane. Such a sequence of membranes permits the extraction process to purify two or more integral membrane proteins separately at different stages (lysates) of the same diafiltration run.

For the extraction of native or recombinantly-expressed, gram-negative inner membrane proteins from bacteria or bacterial host cells containing a recombinant vector, respectively, by differential detergent tangential flow diafiltration, a process is used which comprises:

(a) lysing bacteria or bacterial host cells containing a recombinant vector in a fermentation broth;

(b) diafiltering the lysed fermentation broth from (a) with a buffer which is not retained by the diafiltration membrane, wherein said buffer removes intracellular and extracellular contaminants through the permeate, and

using a chelating agent to prevent proteolysis;

(c) diafiltering the lysate from (b) with a detergent and a buffer which is not retained by the diafiltration membrane, wherein said detergent solubilizes and removes inner membrane proteins, and using a divalent cation to stabilize the outer membrane proteins, thereby preventing their solubilization; and

(d) collecting the inner membrane proteins removed in (c).

For the extraction of native or recombinantly-expressed, gram-negative outer membrane proteins from bacteria or bacterial host cells containing a recombinant vector, respectively, by differential detergent tangential flow diafiltration, a process is used which comprises:

(a) lysing bacteria or bacterial host cells containing a recombinant vector in a fermentation broth;

(b) diafiltering the lysed fermentation broth from (a) with a buffer which is not retained by the diafiltration membrane, wherein said buffer removes intracellular and extracellular contaminants through the permeate, and using a chelating agent to prevent proteolysis;

(c) diafiltering the lysate from (b) with a detegent and a buffer which is not retained by the diafiltration membrane, wherein said detergent solubilizes and removes inner membrane proteins, and using a divalent cation to stabilize the outer membrane proteins, thereby preventing their solubilization;

(d) diafiltering the lysate from (c) with the buffer from (c), and using a divalent cation from (c) in the absence of detergent, in order to reduce the concentration of the detergent from (c);

(e) diafiltering the lysate from (d) with a buffer which is not retained by the diafiltration membrane, a chelating agent and a detergent to solubilize and remove the outer membrane proteins; and

(f) collecting the outer membrane proteins removed in (e).

If desired, further extraction can be performed by adding the following steps to the foregoing process:

(g) diafiltering the lysate from (e) with reagents of (e), with the exception of the detergent, in order to reduce the concentration of the detergent;

(h) diafiltering the lysate from (g) with reagents of (e); and

(i) collecting the outer membrane proteins removed in (h).

For the extraction of lipidated recombinant, outer membrane protein P4 (lipidated rP4) of *H. influenzae* from bacterial host cells by differential detergent tangential flow diafiltration, a process is used which comprises:

(a) lysing bacterial host cells in a fermentation broth;

(b) diafiltering the lysed fermentation broth from (a) with a buffer which is not retained by the diafiltration membrane, wherein said buffer removes intracellular and extracellular contaminants through the permeate, and

using a chelating agent to prevent proteolysis;

(c) diafiltering the lysate from (b) with a detergent and a buffer which is not retained by the diafiltration membrane, wherein said detegent solubilizes and removes inner membrane proteins, and using a divalent cation to stabilize the outer membrane proteins, thereby preventing their solubilization;

(d) diafiltering the lysate from (c) with the buffer from (c), and using a divalent cation from (c) in the absence of detergent, in order to reduce the concentration of the detergent from (c);

(e) diafiltering the lysate from (d) with a buffer which is not retained by the diafiltration membrane, a chelating agent and a detergent to solubilize the outer membrane proteins;

(f) diafiltering the lysate from (e) with a buffer which is not retained by the diafiltration membrane, a chelating agent and a detergent to extract and remove the lipidated rP4; and

(g) collecting the lipidated rP4 removed in (f).

If desired, further extraction of lipidated rP4 can be performed by adding the following steps to the foregoing process:

(h) diafiltering the lysate from (f) with reagents of (f), with the exception of the detergent, in order to reduce the concentration of the detergent;

(i) diafiltering the lysate from (h) with reagents of (f) to extract and remove the lipidated rP4; and

(j) collecting the lipidated rP4 removed in (i).

If also desired, still further extraction of lipidated rP4 can be performed by adding the following steps to the foregoing process:

(k) diafiltering the lysate from (j) with reagents of (f), with the exception of the detergent, in order to reduce the concentration of the detergent;

(l) diafiltering the lysate from (k) with reagents of (f) to extract and remove the lipidated rP4; and

(m) collecting the lipidated rP4 removed in (l).

Still further cycles of lipidated rP4 extraction may be utilized, if desired.

For the extraction of lipidated recombinant, outer membrane protein P6 (lipidated rP6) of *H. influenzae* from bacterial host cells by differential detergent tangential flow diafiltration, a process is used which comprises:

(a) lysing bacterial host cells in a fermentation broth;

(b) diafiltering the lysed fermentation broth from (a) with a buffer which is not retained by the diafiltration membrane, wherein said buffer removes intracellular and extracellular contaminants through the permeate, and using a chelating agent to prevent proteolysis;

(c) diafiltering the lysate from (b) with a detergent and a buffer which is not retained by the diafiltration membrane,

wherein said detergent solubilizes and removes inner membrane proteins, and using a divalent cation to stabilize the outer membrane proteins, thereby preventing their solubilization;

(d) diafiltering the lysate from (c) with a buffer which is not retained by the diafiltration membrane, a chelating agent to sequester divalent cation and to prevent proteolysis, and a detergent to solubilize and remove the outer membrane proteins other than lipidated rP6;

(e) diafiltering the lysate from (d) with a buffer which is not retained by the diafiltration membrane, a chelating agent to prevent proteolysis, a detergent to remove additional outer membrane proteins, and a salt to disrupt the membrane/outer membrane protein complex;

(f) diafiltering the lysate from (e) with reagents of (e), with the exception of the detergent and the salt, in order to reduce the concentration of the detergent;

(g) diafiltering the lysate from (f) with a detergent and a buffer which is not retained by the diafiltration membrane, wherein said detergent solubilizes and removes additional proteins bound to the cellular outer membrane, and using a chelating agent to prevent proteolysis;

(h) diafiltering the lysate from (g) with the buffer from (g) and the chelating agent of (g) to reduce the

concentration of the detergent from (g);

(i) diafiltering the lysate from (h) with a phosphate compound and a detergent to solubilize and remove additional proteins bound to the cellular outer membrane;

(j) diafiltering the lysate from (i) with a phosphate compound to reduce the concentration of the detergent from (i);

(k) heating the lysate from (j) to remove lipidated rP6 from the membrane while diafiltering that lysate with a phosphate compound and a detergent to solubilize, extract and remove the lipidated rP6; and

(l) collecting the lipidated rP6 removed in (k).

If desired, the process for extracting lipidated rP6 may be modified by concentrating the lysate from (j) before proceeding to (k).

In order that this invention may be better understood, the following examples are set forth. The examples are for the purpose of illustration only and are not to be construed as limiting the scope of the invention.

Examples

Example 1
Lipidated rP4 Differential Detergent Membrane Extraction

The overall process for extracting lipidated rP4 from bacterial cells, such as *E. coli* cells,

involved microfluidization or cell lysis and membrane differential detergent extraction. The fermentation broth was harvested and adjusted to 5 mM EDTA to inhibit possible protein degradation from metalloproteases. The broth was then diluted to less than 5% w/v wet cell weight concentration and lysed with a high-pressure microfluidizer (Microfluidics, Newton, MA). The lysed cells were diafiltered with a specific sequence of buffer solutions using a tangential flow system that includes 1000 kD regenerated cellulose Millipore membranes of surface area 0.002 m^2/g wet weight cells. The sequence of buffer solutions was chosen to solubilize inner membrane proteins first and then to solubilize outer membrane proteins which includes rP4. During diafiltration, the solubilized proteins of approximate size less than the molecular weight cut-off of the membrane passed through the permeate, while larger molecules and unsolubilized proteins were retained. The sequence of diafiltration steps was as follows:

(1) The lysed fermentation broth was diafiltered with 10 mM Hepes/1 mM EDTA/pH 8.0 at a volume equal to three times the volume of the retentate to remove intracellular and extracellular contaminants through the permeate.

(2) The lysate was diafiltered five times with 10 mM Hepes/1mM $MgCl_2$ /1% Triton™ X-100, pH 8, to solubilize and remove inner membrane proteins. The Mg^{+2} ions stabilized the outer membrane; therefore, the outer membrane proteins were not solubilized in the presence of Triton™ X-100.

(3) The lysate was diafiltered three times with 10 mM Hepes/1mM $MgCl_2$ /pH 8, to reduce the Triton™ X-100 concentration.

(4) The lysate was diafiltered three times with 50 mM Tris™/5 mM EDTA/1% Zwittergent™ 3-12/pH 8,

to solubilize the outer membrane proteins, including lipidated rP4, and then to begin extracting and collecting lipidated rP4 from the outer membrane.

(5) The lysate was diafiltered three times with 50 mM Tris™/5 mM EDTA, pH 8. This step was performed without Zwittergent™ 3-12, because the Zwittergent™ compounds do not pass through the 1000 kD cut-off membrane as readily as smaller compounds such as salts. This step served to reduce the Zwittergent™ concentration in the membrane; the Zwittergent™ concentration of step (4) would otherwise reduce the flow rate through the membrane during steps (6) and (8) below.

(6) The lysate was diafiltered two times with 50 mM Tris™/5 mM EDTA/1% Zwittergent™ 3-12, pH 8, to continue extracting and collecting lipidated rP4 from the outer membrane.

(7) The lysate was diafiltered two times with 50 mM Tris™/5 mM EDTA, pH 8 to again reduce the Zwittergent™ concentration.

(8) The lysate was diafiltered two times with 50 mM Tris™/5 mM EDTA/1% Zwittergent™ 3-12, pH 8 to continue extracting and collecting lipidated rP4 from the outer membrane.

(9) The lysate was diafiltered two times with 50 mM Tris™/5 mM EDTA, pH 8 to again reduce the Zwittergent™ concentration.

During the diafiltration steps, the transmembrane pressure was maintained at approximately 5 psi and the cross flow rate was maintained at 150 liters/meters^2 membrane area/hour (lmh). The diafiltration processes were run at room temperature. The permeate flux ranged from 30 to 40 lmh, which was sufficiently high for the extraction process to be practical and scalable.

During the extraction, samples were taken at various points for analysis by SDS-PAGE to evaluate

the effect of various diafiltration steps on the extraction of proteins. Samples were precipitated by alcohol addition, centrifuged, and then resolubilized at 20% of the original volume in SDS sample preparation buffer. This method of preparing samples concentrated the sample and reduced the Triton™ or Zwittergent™ 3-12 concentration of the samples. Triton™ X-100 or Zwittergent™ 3-12 interfere with the binding of SDS to the sample and reduce the resolution of bands on gels. Ten μl of each sample was loaded on to Novex (Encinitas, CA) 10% acrylamide gels and the gels were run for 60-90 minutes at 125 Volts.

A typical SDS-PAGE analysis of the samples taken from the permeate streams during the extraction process for lipidated rP4 is shown in Figure 1. Lipidated rP4 ran at approximately 30 kD on these gels. The gel shows that some contaminating proteins were removed during diafiltration with lysis buffer (lane 5) and buffer containing Triton™ X-100 (lane 6). There was very little loss of lipidated rP4 during these diafiltration steps. During the Zwittergent™ 3-12 diafiltration step, lipidated rP4 was extracted in a partially purified state (lane 8). At the end of the Zwittergent™ diafiltration step, very little lipidated rP4 was present in the permeate stream (lane 9). This indicated that most of the solubilized lipidated rP4 had been recovered through the permeate. Other experiments have shown that very little unsolubilized lipidated rP4 remains in the retentate after the completion of the extraction process (data not shown). The 30 kD band of the Zwittergent™ 3-12 extract has been shown to be lipidated rP4 by western analysis (data not shown).

Example 2
Additional Extraction Runs for Lipidated rP4

This Example presents data generated from four additional extraction runs for lipidated rP4. In each run, a recombinant *E. coli* fermentation broth was first adjusted to 5mM EDTA to inhibit possible protein degradation from metalloproteases. The fermentation broth was then adjusted to a wet cell concentration of ten percent and lysed by passing through a Microfluidics microfluidizer. This cell lysate was then aliquoted into portions containing an equivalent of 500 grams of cells and frozen at -70°C.

A 500 gram portion of lysed *E. coli* fermentation broth was then removed from -70°C and thawed in a water bath at a temperature not greater than 40°C. The cell lysate was then diluted to five percent wet cell weight. This five percent cell lysate was then subjected to the differential detergent extraction process utilizing tangential flow diafiltration as described in Example 1. The only slight difference was that in step (4) the diafiltration was conducted twice rather than three times.

SDS-PAGE analysis of samples taken at various points during the extraction gave results comparable to those seen in Figure 1 (data not shown). The amount of lipidated rP4 recovered in each of the four runs was calculated. The percent lipidated rP4 was determined in the cell lysate before and after extraction by running samples on SDS-PAGE gels and then scanning them on a densitometer. This data illustrated that there was an average reduction in grams of 78% of lipidated rP4 protein in the cell lysate before and after extraction. This data also showed that the total recovery of lipidated rP4

protein in the extraction pool as compared to the cell lysate was 18%.

The results are shown in Table 1:

Table 1

Sample	Volume L	Protein mg/ml	Total Protein g	% LrP4	LrP4 g/L	LrP4 g	% Reduction in LrP4	% LrP4 Recovered
N21001 Cell Lysate	10	20.00	200.00	13.70	2.74	27.40		
N21002 Cell Lysate	10	10.80	108.00	12.10	1.31	13.07		
N21003 Cell Lysate	10	11.20	112.00	11.90	1.33	13.33		
N21004 Cell Lysate	10	7.80	78.00	12.60	0.98	9.83		
N21001 Extracted Cell Lysate	10	2.60	26.00	14.80	0.38	3.85	85.96%	14.04%
N21002 Extracted Cell Lysate	10	1.30	13.00	12.40	0.16	1.61	87.66%	12.34%
N21003 Extracted Cell Lysate	10	1.20	12.00	13.40	0.16	1.61	87.94%	12.06%
N21004 Extracted Cell Lysate	10	3.40	34.00	14.30	0.49	4.86	50.53%	49.47%
N21001 Extraction Pool	120	0.06	7.20	56.30	0.03	4.05		14.79%
N21002 Extraction Pool	120	0.06	6.60	42.30	0.02	2.79		21.36%
N21003 Extraction Pool	120	0.03	3.12	73.60	0.02	2.30		17.23%
N21004 Extraction Pool	120	0.05	6.24	29.90	0.02	1.87		18.98%

The reproducibility of the diafiltration process is illustrated in Figure 2. The permeate flux from the four runs over the course of the extraction process was monitored. The similarity in the flux rates throughout the process demonstrates that the extraction process is controllable and reproducible.

To purify the extracted lipidated rP4, the lipidated rP4-containing cell lysate extract was then processed through tandem ion exchange columns consisting of a DEAE Sepharose™ Fast Flow and a SP

Sepharose™ Fast Flow column (Pharmacia & Upjohn, Piscataway, NJ). The columns were washed with additional equilibration buffer and the DEAE column was then removed from the process stream. The SP column was then washed with 20 column volumes of equilibration buffer and then eluted with a NaCl step gradient, yielding purified LrP4 30K protein. The 20mM NaCl concentration eluted a purified aggregated form of lipidated rP4 protein (Form I). The 140mM NaCl concentration eluted a mixture of aggregated and non-aggregated form of lipidated rP4 protein (Form II).

The Form II lipidated rP4 30K protein was then converted to the more aggregated Form I state by subjecting the protein to a controlled slow freezing. The two purified forms may be purified and stored separately, or may be purified separately and then combined. The conversion process was as follows:

(1) Obtain sterile filtered aliquots of lipidated rP4 Form II.

(2) Slow freeze to -6°C.

Example 3

Lipidated rP6 Differential Detergent Membrane Extraction

The process for extracting lipidated rP6 was similar to the process for extracting lipidated rP4. However, the diafiltration process required more steps because lipidated rP6 is tightly associated with peptidoglycans. The fermentation broth of *E. coli* cells expressing lipidated rP6 was adjusted to 10 mM EDTA and diluted to less than or equal to 10% wet weight cells/volume prior to homogenization. The cells were then lysed with a high-pressure microfluidizer and diafiltered at room temperature with a sequence of buffers using a cross-flow membrane

filtration device. It was determined that the minimum membrane area to allow efficient mass transport of solubilized proteins through the membrane was approximately 0.002 m^2/g wet weight cells. The solubilized proteins of approximate size less than the 1000 kD molecular weight cut-off rating of the membrane passed through with the permeate, while larger molecules and unsolubilized proteins were retained. The sequence of diafiltration steps was as follows:

(1) The lysed fermentation broth was diafiltered with 10 mM Hepes/1 mM EDTA/pH 8.0 (lysis buffer) at a volume equal to three times the volume of the retentate to remove intracellular and extracellular contaminants through the permeate.

(2) The lysate was diafiltered three times with 10 mM Hepes/1mM $MgCl_2$ /0.2% Triton™ X-100 to solubilize and remove inner membrane proteins. The Mg^{+2} ions stabilized the outer membrane; therefore, the outer membrane proteins were not solubilized in the presence of Triton™ X-100.

(3) The lysate was diafiltered three times with 50 mM Tris™ /5 mM EDTA/0.2% Zwittergent™ 3-14 to solubilize and remove other outer membrane proteins (but not lipidated rP6). The EDTA serves to sequester the Mg^{+2} ions from step (2), as well as to prevent proteolysis.

(4) The lysate was diafiltered three times with 50 mM Tris™/5 mM EDTA/0.5 M NaCl/0.2% Zwittergent™ 3-14 to solubilize and remove additional proteins. NaCl was added to the buffer in this step to disrupt any ionic interactions between membrane proteins and membranes. This step was performed because lipidated rP6 is a peptidoglycan-associated lipoprotein, and the salt serves to remove membrane-bound proteins (but not lipidated rP6) from the membrane/outer membrane protein complex (lipidated

rP4 is not so associated; thus this step was not performed for extracting that protein). The diafiltration was continued with three retentate volumes of 50 mM Tris™/5mM EDTA to reduce the Zwittergent™ concentration in the retentate.

(5) The lysate was diafiltered three times with 50 mM Tris™ /5 mM EDTA/0.2% sarcosyl to remove additional membrane-bound proteins (but not lipidated rP6) and then diafiltered three times with 50 mM Tris™ /5 mM EDTA to reduce the sarcosyl concentration in the retentate.

(6) The lysate was diafiltered three times with 10 mM sodium phosphate/0.2% Zwittergent™ 3-12 to remove additional membrane-bound proteins (but not lipidated rP6), and then diafiltered three times with 10 mM sodium phosphate to reduce the Zwittergent™ 3-12 concentration in the retentate.

(7) The lysate was concentrated to 20% of its original volume and then diafiltered three times with 10 mM sodium phosphate/0.2% Zwittergent™ 3-12 at 55°C to solubilize lipidated rP6, which was collected through the permeate. The concentration was performed prior to diafiltration to increase the concentration of lipidated rP6 in the permeate. The diafiltration was continued for three additional retentate volumes with 10 mM sodium phosphate at 55°C to reduce the Zwittergent™ 3-12 concentration in the retentate. This heating step was performed because (as in step (4) above)) lipidated rP6 is a peptidoglycan-associated lipoprotein, and heating serves to remove lipidated rP6 from the membrane/membrane protein complex (lipidated rP4 is not so associated; thus this step was not performed for extracting that protein). Finally, the diafiltration was concluded with three retentate volumes of 10 mM sodium phosphate at 55°C.

During the diafiltration steps, the transmembrane pressure was maintained at approximately

10 psi and the cross flow rate was maintained at approximately 120-180 lmh. All the diafiltration processes were run at room temperature, except the final 55°C extraction step, which was run at the higher temperature to solubilize lipidated rP6. The permeate flux ranged from 30 to 50 lmh, which was sufficiently high for the extraction process to be practical and scalable.

During the extraction, samples were taken at various points for analysis by SDS-PAGE to evaluate the effect of various diafiltration steps on the extraction of proteins. Samples were prepared and run on gels as described in Example 1.

A typical SDS-PAGE analysis of the samples taken from the permeate streams during the extraction process of lipidated rP6 is shown in Figures 3 and 4. Lipidated rP6 ran at approximately 15 kD on these gels. The gels showed that some contaminating proteins are removed during diafiltration with the lysis buffer (Figure 3, lane 2) and buffer containing various detergents (Figure 3, lanes 5-6 and Figure 4, lane 3). There was very little loss of lipidated rP6 during these diafiltration steps. During the final Zwittergent™ 3-12 diafiltration step at 55°C, lipidated rP6 was extracted in a partially purified state (Figure 4, lane 6). At the end of the second Zwittergent™ 3-12 diafiltration step at 55°C, very little lipidated rP6 was present in the permeate stream (Figure 4, lane 8). This suggested that most of the solubilized lipidated rP6 had been recovered through the permeate. Other experiments have shown that very little lipidated rP6 remains unsolubilized in the retentate after the completion of the diafiltration process (data not shown). The 15 kD band of the Zwittergent™ 3-12 /55°C extract has been shown to be lipidated rP6 by western analysis (data not shown).

The process described above was repeated to extract lipidated rP6 from the broth of another *E. coli* fermentation. The SDS-PAGE analysis was similar to that seen in Figures 3 and 4 (data not shown), and the 15 kD band of the Zwittergent™ 3-12 /55°C extract was again shown to be lipidated rP6 by western analysis (data not shown).

Example 4
Additional Extraction Runs for Lipidated rP6

This Example presents data generated from three additional extraction runs for lipidated rP6. In each run, the process described in Example 3 was used to extract lipidated rP6 from a recombinant *E. coli* fermentation broth.

The results are shown in Table 2:

Table 2

Sample (15ml)	Volume L	Protein mg/ml	Total Protein g	Purity %	Total LrP6 g
N4-1002 Lysed Fermentation Broth	5	18.70	93.50	10.70%	10.00
N4-1002 55C Extraction Pool	20	0.17	3.43	34.20%	1.17
N4-1003 Lysed Fermentation Broth	5	14.74	73.71	2.30%	1.70
N4-1003 55C Extraction Pool	20	0.73	14.52	19.50%	2.83
N4-1004 Lysed Fermentation Broth	5	14.74	73.71	7.20%	5.31
N4-1004 55C Extraction Pool	20	0.08	1.66	26.00%	0.43

What is claimed is:

1. A process for extracting native or recombinantly-expressed, gram-negative inner membrane proteins from bacteria or bacterial host cells containing a recombinant vector by differential detergent tangential flow diafiltration, which comprises:

(a) lysing bacteria or bacterial host cells containing a recombinant vector in a fermentation broth;

(b) diafiltering the lysed fermentation broth from (a) with a buffer which is not retained by the diafiltration membrane, wherein said buffer removes intracellular and extracellular contaminants through the permeate, and using a chelating agent to prevent proteolysis;

(c) diafiltering the lysate from (b) with a detergent and a buffer which is not retained by the diafiltration membrane, wherein said detergent solubilizes and removes inner membrane proteins, and using a divalent cation to stabilize the outer membrane proteins, thereby preventing their solubilization; and

(d) collecting the inner membrane proteins removed in (c).

2. The process of Claim 1 wherein: the lysis of (a) occurs in a microfluidizer; in (b), the buffer is selected from the group consisting of Hepes, 3-(N-morpholino)propane sulfonic acid (MOPS), Tris™, sodium phosphate and sodium borate; and in (c), the buffer is selected from the group consisting of Hepes, MOPS, Tris™, sodium phosphate and sodium borate, the detergent is selected from the group consisting of a

Zwittergent™ compound, a non-ionic Triton™ compound, sarcosyl, a glucoside compound, a cholate compound and dodecyl-maltoside, and the divalent cation is selected from the group consisting of Mg^{+2} and Ca^{+2}.

3. The process of Claim 2 wherein in (b), the buffer is Hepes and the chelating agent is EDTA; and in (c), the buffer is Hepes, the detergent is Triton™ X-100, and the divalent cation is Mg^{+2}.

4. A process for extracting native or recombinantly-expressed, gram-negative outer membrane proteins from bacteria or bacterial host cells containing a recombinant vector by differential detergent tangential flow diafiltration, which comprises:

(a) lysing bacteria or bacterial host cells containing a recombinant vector in a fermentation broth;

(b) diafiltering the lysed fermentation broth from (a) with a buffer which is not retained by the diafiltration membrane, wherein said buffer removes intracellular and extracellular contaminants through the permeate, and using a chelating agent to prevent proteolysis;

(c) diafiltering the lysate from (b) with a detergent and a buffer which is not retained by the diafiltration membrane, wherein said detergent solubilizes and removes inner membrane proteins, and using a divalent cation to stabilize the outer membrane proteins, thereby preventing their solubilization;

(d) diafiltering the lysate from (c) with the buffer from (c), and using a divalent cation from (c) in the absence of detergent, in order to reduce the

concentration of the detergent from (c);

(e) diafiltering the lysate from (d) with a buffer which is not retained by the diafiltration membrane, a chelating agent and a detergent to solubilize and remove the outer membrane proteins; and

(f) collecting the outer membrane proteins removed in (e).

5. The process of Claim 4 wherein: the lysis of (a) occurs in a microfluidizer; in (b), the buffer is selected from the group consisting of Hepes, MOPS, Tris™, sodium phosphate and sodium borate; in (c), the buffer is selected from the group consisting of Hepes, MOPS, Tris™, sodium phosphate and sodium borate, the detergent is selected from the group consisting of Zwittergent™ compound, a non-ionic Triton™ compound, sarcosyl, a glucoside compound, a cholate compound and dodecyl-maltoside, and the divalent cation is selected from the group consisting of Mg^{+2} and Ca^{+2}; in (d), the buffer is selected from the group consisting of Hepes, MOPS, Tris™, sodium phosphate and sodium borate, and the divalent cation is selected from the group consisting of Mg^{+2} and Ca^{+2}; and in (e), the buffer is selected from the group consisting of Hepes, MOPS, Tris™, sodium phosphate and sodium borate, and the detergent is selected from the group consisting of Zwittergent™ compound, a non-ionic Triton™ compound, sarcosyl, a glucoside compound, a cholate compound and dodecyl-maltoside.

6. The process of Claim 3 wherein in (b), the buffer is Hepes and the chelating agent is EDTA; in (c), the buffer is Hepes, the detergent is Triton™ X-100, and the divalent cation is Mg^{+2}; in (d), the buffer is Hepes and the divalent cation is Mg^{+2}; and in (e), the buffer is Tris™, the chelating agent is EDTA, and the detergent is Zwittergent™ 3-12.

7. The process of Claim 4, which further comprises:

(g) diafiltering the lysate from (e) with reagents of (e), with the exception of the detergent, in order to reduce the concentration of the detergent;

(h) diafiltering the lysate from (g) with reagents of (e); and

(i) collecting the outer membrane proteins removed in (h).

8. A process for extracting lipidated recombinant outer membrane protein P4 (rP4) of *Haemophilus influenzae* from bacterial host cells by differential detergent tangential flow diafiltration, which comprises:

(a) lysing bacterial host cells in a fermentation broth;

(b) diafiltering the lysed fermentation broth from (a) with a buffer which is not retained by the diafiltration membrane, wherein said buffer removes intracellular and extracellular contaminants through the permeate, and using a chelating agent to prevent proteolysis;

(c) diafiltering the lysate from (b) with a detergent and a buffer which is not retained by the diafiltration membrane, wherein said detergent solubilizes and removes inner membrane proteins, and using a divalent cation to stabilize the outer membrane proteins, thereby preventing their solubilization;

(d) diafiltering the lysate from (c) with the buffer from (c), and using a divalent cation from (c) in the absence of detergent, in order to reduce the

concentration of the detergent from (c);

(e) diafiltering the lysate from (d) with a buffer which is not retained by the diafiltration membrane, a chelating agent and a detergent to solubilize the outer membrane proteins;

(f) diafiltering the lysate from (e) with a buffer which is not retained by the diafiltration membrane, a chelating agent and a detergent to extract and remove the lipidated rP4; and

(g) collecting the lipidated rP4 removed in (f).

9. The process of Claim 8 wherein: the lysis of (a) occurs in a microfluidizer; in (b), the buffer is selected from the group consisting of Hepes, MOPS, Tris™, sodium phosphate and sodium borate; in (c), the buffer is selected from the group consisting of Hepes, MOPS, Tris™, sodium phosphate and sodium borate, the detergent is selected from the group consisting of Zwittergent™ compound, a non-ionic Triton™ compound, sarcosyl, a glucoside compound, a cholate compound and dodecyl-maltoside, and the divalent cation is selected from the group consisting of Mg^{+2} and Ca^{+2}; in (d), the buffer is selected from the group consisting of Hepes, MOPS, Tris™, sodium phosphate and sodium borate, and the divalent cation is selected from the group consisting of Mg^{+2} and Ca^{+2}; in (e), the buffer is selected from the group consisting of Hepes, MOPS, Tris™, sodium phosphate and sodium borate, and the detergent is selected from the group consisting of Zwittergent™ compound, a non-ionic Triton™ compound, sarcosyl, a glucoside compound, a cholate compound and dodecyl-maltoside; and in (f), the buffer is selected from the group consisting of Hepes, MOPS, Tris™, sodium phosphate and sodium

borate, and the detergent is selected from the group consisting of Zwittergent™ compound, a non-ionic Triton™ compound, sarcosyl, a glucoside compound, a cholate compound and dodecyl-maltoside.

10. The process of Claim 9 wherein in (b), the buffer is Hepes and the chelating agent is EDTA; in (c), the buffer is Hepes, the detergent is Triton™ X-100, and the divalent cation is Mg^{+2}; in (d), the buffer is Hepes and the divalent cation is Mg^{+2}; in (e), the buffer is Tris™, the chelating agent is EDTA, and the detergent is Zwittergent™ 3-12; and in (f), the buffer is Tris™, the chelating agent is EDTA, and the detergent is Zwittergent™ 3-12.

11. The process of Claim 8, which further comprises:

(h) diafiltering the lysate from (f) with reagents of (f), with the exception of the detergent, in order to reduce the concentration of the detergent;

(i) diafiltering the lysate from (h) with reagents of (f) to extract and remove the lipidated rP4; and

(j) collecting the lipidated rP4 removed in (i).

12. The process of Claim 11, which further comprises:

(k) diafiltering the lysate from (j) with reagents of (f), with the exception of the detergent, in order to reduce the concentration of the detergent;

(l) diafiltering the lysate from (k) with reagents of (f) to extract and remove the lipidated rP4; and

(m) collecting the lipidated rP4 removed in (l).

13. A process for extracting lipidated recombinant outer membrane protein P6 (rP6) of

Haemophilus influenzae from bacterial host cells by differential detergent tangential flow diafiltration, which comprises:

(a) lysing bacterial host cells in a fermentation broth;

(b) diafiltering the lysed fermentation broth from (a) with a buffer which is not retained by the diafiltration membrane, wherein said buffer removes intracellular and extracellular contaminants through the permeate, and using a chelating agent to prevent proteolysis;

(c) diafiltering the lysate from (b) with a detergent and a buffer which is not retained by the diafiltration membrane, wherein said detergent solubilizes and removes inner membrane proteins, and using a divalent cation to stabilize the outer membrane proteins, thereby preventing their solubilization;

(d) diafiltering the lysate from (c) with a buffer which is not retained by the diafiltration membrane, a chelating agent to sequester divalent cation and to prevent proteolysis, and a detergent to solubilize and remove the outer membrane proteins other than lipidated rP6;

(e) diafiltering the lysate from (d) with a buffer which is not retained by the diafiltration membrane, a chelating agent to prevent proteolysis, a detergent to remove additional outer membrane proteins, and a salt to disrupt the membrane/outer membrane protein complex;

(f) diafiltering the lysate from (e) with reagents of (e), with the exception of the detergent and the salt, in order to reduce the concentration of the detergent;

(g) diafiltering the lysate from (f) with a detergent and a buffer which is not retained by the diafiltration membrane, wherein said detergent solubilizes and removes additional proteins bound to the cellular outer membrane, and using a chelating agent to prevent proteolysis;

(h) diafiltering the lysate from (g) with the buffer from (g) and the chelating agent of (g) to reduce the concentration of the detergent from (g);

(i) diafiltering the lysate from (h) with a phosphate compound and a detergent to solubilize and remove additional proteins bound to the cellular outer membrane;

(j) diafiltering the lysate from (i) with a phosphate compound to reduce the concentration of the detergent from (i);

(k) heating the lysate from (j) to remove lipidated rP6 from the membrane while diafiltering that lysate with a phosphate compound and a detergent to solubilize, extract and remove the lipidated rP6; and

(l) collecting the lipidated rP6 removed in (k).

14. The process of Claim 13 wherein: the lysis of (a) occurs in a microfluidizer; in (b), the

buffer is selected from the group consisting of Hepes, MOPS, Tris™, sodium phosphate and sodium borate, and the divalent cation is selected from the group consisting of Mg^{+2} and Ca^{+2}; in (c), the buffer is selected from the group consisting of Hepes, MOPS, Tris™, sodium phosphate and sodium borate, the detergent is selected from the group consisting of Zwittergent™ compound, a non-ionic Triton™ compound, sarcosyl, a glucoside compound, a cholate compound and dodecyl-maltoside, and the divalent cation is selected from the group consisting of Mg^{+2} and Ca^{+2}; in (d), the buffer is selected from the group consisting of Hepes, MOPS, Tris™, sodium phosphate and sodium borate, and the detergent is selected from the group consisting of Zwittergent™ compound, a non-ionic Triton™ compound, sarcosyl, a glucoside compound, a cholate compound and dodecyl-maltoside; in (e), the buffer is selected from the group consisting of Hepes, MOPS, Tris™, sodium phosphate and sodium borate, the salt is a sodium salt, and the detergent is selected from the group consisting of Zwittergent™ compound, a non-ionic Triton™ compound, sarcosyl, a glucoside compound, a cholate compound and dodecyl-maltoside; in (f), the buffer is selected from the group consisting of Hepes, MOPS, Tris™, sodium phosphate and sodium borate; in (g), the buffer is selected from the group consisting of Hepes, MOPS, Tris™, sodium phosphate and sodium borate, and the detergent is selected from the group consisting of Zwittergent™ compound, a non-ionic Triton™ compound, sarcosyl, a glucoside compound, a cholate compound and dodecyl-maltoside; in (h), the buffer is selected from the group consisting of Hepes, MOPS, Tris™, sodium phosphate and sodium borate; in (i), the detergent is selected from the group consisting of Zwittergent™ compound, a non-ionic Triton™ compound, sarcosyl, a glucoside compound, a cholate compound and dodecyl-maltoside; and in (k),

the detergent is selected from the group consisting of Zwittergent™ compound, a non-ionic Triton™ compound, sarcosyl, a glucoside compound, a cholate compound and dodecyl-maltoside.

15. The process of Claim 14 wherein in (b), the buffer is Hepes and the chelating agent is EDTA; in (c), the buffer is Hepes, the detergent is Triton™ X-100, and the divalent cation is Mg^{+2}; in (d), the buffer is Hepes, the chelating agent is EDTA, and the detergent is Zwittergent™ 3-14; in (e), the buffer is Hepes, the chelating agent is EDTA, the salt is sodium chloride, and the detergent is Zwittergent™ 3-14; in (f), the buffer is Tris™ and the chelating agent is EDTA; in (g), the buffer is Tris™, the detergent is sarcosyl, and the chelating agent is EDTA; in (h), the buffer is Tris™ and the chelating agent is EDTA; in (i), the detergent is Zwittergent™ 3-12, and the phosphate is sodium phosphate; in (j), the phosphate is sodium phosphate; and in (k), the detergent is Zwittergent™ 3-12.

16. The process of Claim 13 wherein prior to (k), the lysate from (j) is concentrated.

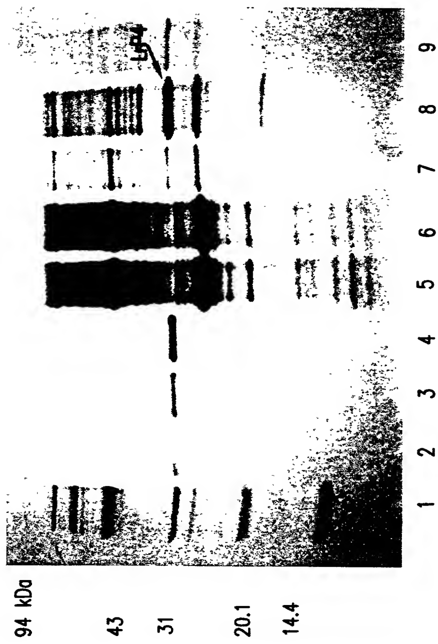

FIG.1

10/019163

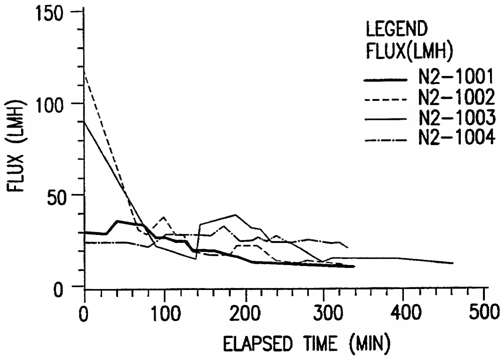

FIG.2

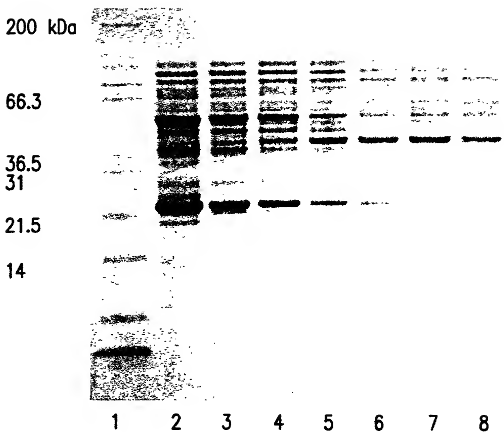

FIG.3

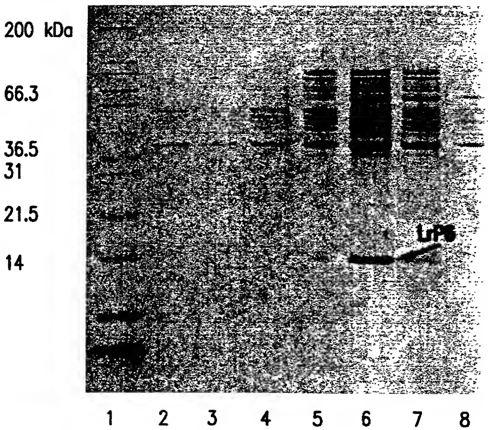

FIG.4

COMBINED DECLARATION AND POWER OF ATTORNEY
(Original, Design, Supplemental, Divisional, Continuation,CIP)

As the below named inventor, I hereby declare that:

INVENTORSHIP IDENTIFICATION

My residence, post office address and citizenship are as stated below next to my name. I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

TITLE OF INVENTION

Extraction of Integral Membrane Proteins

SPECIFICATION IDENTIFICATION

the specification of which: (complete (a), (b), or (c))

(a) ☒ was described and claimed in PCT International Application No. PCT/US00/17019 filed on June 20, 2000, which is now entering the U.S. National Stage.

(b) ☐ was filed on as
☐ Serial Number
☐ Express Mail No. , as Serial Number not yet known

(c) ☐ was described and claimed in PCT International Application No. filed on and as amended under PCT Article 19 on (if any).

ACKNOWLEDGEMENT OF REVIEW OF PAPERS AND DUTY OF CANDOR

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to the patentability of this application in accordance with Title 37 CFR 1.56(a).

PRIORITY CLAIM

I hereby claim foreign priority benefits under Title 35, United States Code, Section 119 of any foreign application(s) for patent or inventors certificate or of any PCT International application(s) designating at least one country other than the United States of America listed below and have also identified below any foreign application(s) for patent or inventor's certificate of any PCT International application(s) designating at least one country other than the United States of America filed by me on the same subject matter having a filing date before that of the application(s) on which priority is claimed.

(d) ☒ No such applications have been filed.
(e) ☐ Such applications have been filed as follows.

NOTE: Where item (c) is entered above and the International Application which designated the U.S. claimed priority, check item (e), enter the details below and make the priority claim.

Earliest Foreign Application(s), if any, filed within 12 months (6 months for Design) prior to this U.S. Application

Country	Application No.	Date of Filing (Day, Month, Year)	Priority Claimed 35 USC 119

All Foreign Application(s), if any, Filed More Than 12 Months (6 Months for Design) Prior to This U.S. Application)

CLAIM FOR BENEFIT OF PRIOR U.S. PROVISIONAL APPLICATION(S)
(35 U.S.C. § 119(E))

I hereby claim the benefit under Title 35, United States Code, § 119(e) of any United States provisional application(s) listed below:

PROVISIONAL APPLICATION NUMBER	FILING DATE
60/141,067	June 25, 1999

CLAIM FOR BENEFIT OF EARLIER U.S./PCT APPLICATION(S)
(UNDER 35 U.S.C. 120)

I hereby claim the benefit under Title 35, United States Code, Section 120 of any United States application(s) designating the United States of America that is/are listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in that/those prior application(s) in the manner provided by the first paragraph of Title 35, United States Code, Section 112, I acknowledge the duty to disclose material information as defined in Title 37, Code of Federal Regulations, Section 1.56(a) which occurred between the filing date of the prior application(s) and the national or PCT International filing date of this application.

PRIOR U.S. APPLICATIONS OR PCT INTERNATIONAL APPLICATIONS DESIGNATING THE U.S. FOR BENEFIT UNDER 35 USC 120

U.S. Applications		**Status (Check One)**		
U.S. Applications	U.S. Filing Date	Patented	Pending	Abandoned
		☐	☐	☐
		☐	☐	☐
		☐	☐	☐
		☐	☐	☐

PCT Applications Designating U.S.		
PCT APPLICATION NO.	PCT FILING DATE	U.S. SERIAL NO. ASSIGNED (if any)
PCT/US00/17019	6/20/00	

POWER OF ATTORNEY

As a named inventor, I hereby appoint the following attorney(s) and/or agent(s) to prosecute this application and transact all business in the Patent and Trademark Office connected therewith.

☒ Customer Number: 25291

Bar Code:

25291
PATENT TRADEMARK OFFICE

☐ Attached as part of this declaration and power of attorney is the authorization of the above-named attorney(s) to accept and follow instructions from my representative(s).

SEND CORRESPONDENCE TO:
Customer Number: 25291

Bar Code:

25291
PATENT TRADEMARK OFFICE

DIRECT ALL TELEPHONE CALLS TO:
Name: Alan M. Gordon
Tel. No. (845) 602-4636

DECLARATION

I hereby declare that all statements herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements are made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

SIGNATURE(S)

Full name of SOLE OR FIRST INVENTOR: **Sanjay Lakhotia**

Inventor's Signature Sanjay Lakhotia Date 12/4/01

Country of Citizenship: **United States of America**
Residence : **42 Wood Ranch Circle, Danville, California 94506** CA
Post Office Address: **42 Wood Ranch Circle, Danville, California 94506**

Full name of SECOND JOINT INVENTOR: **Michael R. Biehl**

Inventor's Signature ______________ Date ______________

Country of Citizenship: **United States of America**
Residence : **1603 Crepe Myrtle Drive, Sanford, North Carolina 27330** NC
Post Office Address: **1603 Crepe Myrtle Drive, Sanford, North Carolina 27330**

Full name of THIRD JOINT INVENTOR:

Inventor's Signature ______________ Date ______________

Country of Citizenship:
Residence :
Post Office Address:

Full name of FOURTH JOINT INVENTOR:

Inventor's Signature ______________ Date ______________

Country of Citizenship:
Residence :
Post Office Address:

COMBINED DECLARATION AND POWER OF ATTORNEY
(Original, Design, Supplemental, Divisional, Continuation,CIP)

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Extraction of Integral Membrane Proteins

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the specification of which: (complete (a), (b), or (c))

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Country	Application No.	Date of Filing (Day, Month, Year)	Priority Claimed 35 USC 119

All Foreign Application(s), if any, Filed More Than 12 Months
(6 Months for Design) Prior to This U.S. Application)

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(UNDER 35 U.S.C. 120)

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PRIOR U.S. APPLICATIONS OR PCT INTERNATIONAL APPLICATIONS DESIGNATING THE U.S. FOR BENEFIT UNDER 35 USC 120

U.S. Applications		**Status (Check One)**		
U.S. Applications	U.S. Filing Date	Patented	Pending	Abandoned
		☐	☐	☐
		☐	☐	☐
		☐	☐	☐
		☐	☐	☐

PCT Applications Designating U.S.		
PCT APPLICATION NO.	PCT FILING DATE	U.S. SERIAL NO. ASSIGNED (if any)
PCT/US00/17019	6/20/00	

POWER OF ATTORNEY

As a named inventor, I hereby appoint the following attorney(s) and/or agent(s) to prosecute this application and transact all business in the Patent and Trademark Office connected therewith.

☒ Customer Number: 25291 Bar Code: 25291 PATENT TRADEMARK OFFICE

☐ Attached as part of this declaration and power of attorney is the authorization of the above-named attorney(s) to accept and follow instructions from my representative(s).

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Name: Alan M. Gordon
Tel. No. (845) 602-4636

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SIGNATURE(S)

Full name of SOLE OR FIRST INVENTOR: **Sanjay Lakhotia**

Inventor's Signature ____________________ Date ____________________

Country of Citizenship: **United States of America**
Residence : **42 Wood Ranch Circle, Danville, California 94506**
Post Office Address: **42 Wood Ranch Circle, Danville, California 94506**

Full name of SECOND JOINT INVENTOR: **Michael R. Biehl**

Inventor's Signature Michael R. Biehl Date 12-13-01

Country of Citizenship: **United States of America**
Residence : **1603 Crepe Myrtle Drive, Sanford, North Carolina 27330**
Post Office Address: **1603 Crepe Myrtle Drive, Sanford, North Carolina 27330**

Full name of THIRD JOINT INVENTOR:

Inventor's Signature ____________________ Date ____________________

Country of Citizenship:
Residence :
Post Office Address:

Full name of FOURTH JOINT INVENTOR:

Inventor's Signature ____________________ Date ____________________

Country of Citizenship:
Residence :
Post Office Address: